Ornithine Decarboxylase Levels in Patients with Normal Colonic Mucosa¹⁾

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Summary: We describe a systematic examination of ornithine decarboxylase activity in 120 colonic mucosal samples which were obtained from 20 subjects without colonic disease to establish the normal mean and standard deviation from proximal to distal colon. Ornithine decarboxylase activity was determined by releasing CO₂ from DL-[1-¹⁴C]ornithine. The mean ornithine decarboxylase levels (CO₂ liberated) ranged from 0.26 ± 0.08 nmol/h · mg protein in the caecum to 0.44 ± 0.16 nmol/h · mg protein in the rectum. There was no difference between sex and age. Ornithine decarboxylase was not stimulated by guanosine 5'-triphosphate. α-Difluoromethylornithine showed an ornithine decarboxylase inhibition of 97.1%. Ornithine decarboxylase activity can be measured with reliable precision and reproducibility. The knowledge of the normal range of ornithine decarboxylase activity in normal human colonic mucosa is necessary for the determination of ornithine decarboxylase activity in pathological findings, especially in malignant transformation.

Introduction

Ornithine decarboxylase²⁾ catalyzes the first step in the biosynthesis of polyamines with the formation of putrescine and CO₂ from L-ornithine utilizing pyridoxal-5-phosphate as a cofactor (1–3). Ornithine decarboxylase is a highly regulated enzyme with a very short in vivo half-life of 10–20 minutes (4). It is a key enzyme in the regulation of growth processes and cell proliferation (5). Ornithine decarboxylase is a very minor component of the total soluble protein in most cells and can be induced by a large variety of growth-promoting stimuli such as hormones, growth factors, tissue regeneration activities, drugs and bile acids (6–10). There are existing multiple isoforms of ornithine decarboxylase in mammalian cells (11), of which one isoform can be stimulated by guanosine 5'-triphosphate (GTP) (12, 13).

Numerous studies have demonstrated increased and decreased ornithine decarboxylase activities in benign colonic adenomas (14–17) and in colon carcinomas (17–23). Ornithine decarboxylase levels have been shown to differ as much as 2–100 fold between different laboratories (24–26). Studies published until now have not systematically investigated the range of normal levels of ornithine decarboxylase in subjects without or-

ganic intestinal diseases. Without these data, however, the interpretation of ornithine decarboxylase assays in pathological colonic mucosa remains unclear and its use to detect possible malignant transformation must be questionable.

In order to determine a reference range of ornithine decarboxylase activity in different parts of the colon we examined ornithine decarboxylase levels in normally appearing colonic mucosa in male and female subjects of different ages.

Materials and Methods

Patients

Twenty patients (11 women, 9 men) aged 17–78 (mean 47.7) years were included in the study. All patients had unclear abdominal symptoms and underwent total colonoscopy. Organic intestinal diseases were excluded. There was also no evidence for systemic diseases such as carcinomas, diabetes, infections, hepatic or renal insufficiency. Blood tests as haemoglobin, erythrocyte sedimentation rate and leukocyte count were normal and there was no history of prior colonic disease. Informed consent was obtained from all subjects according to the Helsinki Declaration.

Biopsy procedures

The colonic cleansing was performed with senna and Golytely lavage as previously described by Ziegenhagen et al. (27). Biopsies were taken from the caecum, ascending, transverse, descending, sigmoid and rectum by a biopsy forceps (Olympus FB-24Q). Tissue biopsies were placed immediately in liquid nitrogen for ornithine decarboxylase analyses and in formaldehyde for microscopical examination. These biopsies for ornithine decarboxylase analyses were subsequently stored at –80 °C until the assay was done during the next three weeks.

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²⁾ Enzymes:

Ornithine decarboxylase (EC 4.1.1.17)

Ornithine-2-oxo-acid aminotransferase (EC 2.6.1.13)

The biopsies were considered to be "normal" when either the endoscopist found normally appearing mucosa and the pathologist did not find any pathological alterations.

Assays

Homogenization and the assay for ornithine decarboxylase were done as a slight modification of the method of *Haarstad* et al. (28). The specimens were weighed and homogenized by an ice-cooled Dounce glass/glass homogenizer 1 g/20 ml in homogenizing buffer consisting of 50 mmol/l Tris buffer (pH 7.5), 0.2 mmol/l pyridoxal-5-phosphate, 0.5 mmol/l ethylenediaminetetraacetate (EDTA), 2 mmol/l dithiothreitol, 0.2 mmol/l freshly added phenylmethylsulphonyl fluoride. The homogenates were centrifuged at 46 320 g (Heraeus Biofuge 28 RS) for 30 min at 4 °C. The supernatant was immediately mixed with an equal volume of storage buffer (glycerol, volume fraction 0.84, 1 mmol/l EDTA, 0.4 mmol/l pyridoxal-5-phosphate and stored at -20 °C for subsequent analyzes. This cytosol-glycerol fraction was assayed for ornithine decarboxylase activity (protein content 1 g/l).

In 5 specimens an aliquot of the homogenate was also centrifuged for 1 hour at 100 000 g in the ultracentrifuge (Beckman Optima L) and the supernatant was mixed with storage buffer (1 + 1).

Also in 5 specimens an aliquot of the 46 320 g supernatant was dialyzed overnight at 4 °C against 100 volumes of homogenization buffer.

Ornithine decarboxylase activity was measured by the release of $^{14}\text{CO}_2$ from *DL*-[1- ^{14}C]ornithine as previously described by *Russell & Snyder* (1) and modified by *Haarstad* et al. (28) and *Löser* et al. (29). All measurements were done twice in a tight fitting rubber capped siliconized test tube, fitted with a syringe.

The standard assay mixture contained in a total reaction volume of 200 µl: 30 µl cytosol-glycerol fraction (containing 28–32 µg protein), 18.5 KBq (0.5 µCi) *DL*-[1- ^{14}C]ornithine hydrochloride (2094.2 GBq/mol $\pm$ 56.6 mCi/mmol) Amersham-Buchler, Braunschweig, Germany, Lot-No: 69) and final concentrations of 46 mmol/l Tris pH 7.5, 0.538 mmol/l EDTA, 1.85 mmol/l dithiothreitol and 0.215 mmol/l pyridoxal-5-phosphate. The released $^{14}\text{CO}_2$ was trapped by a piece of Whatman filter paper (No. 1MM, 0.65 mm²) impregnated with 25 µl of the CO_2 trapping agent hyamine hydroxide (1 mol/l in methanol) (Sigma Inc., Munich). After an incubation time of 60 min at 37 °C in a shaking bath the reaction was stopped by injection of 200 µl 6 mol/l perchloric acid through the rubber stopper. The incubation was continued routinely for another 30 min at 37 °C to ensure complete absorption of $^{14}\text{CO}_2$. In 22 cases the incubation was continued for 60 min. The radioactivity on the filter papers was counted with a β -counter Wallac 1410 (Pharmacia). To avoid chemiluminescence, the papers were counted 24 hours later standing at room temperature in the dark in 10 ml Quickszint 1 (Zinsser Analytic, Frankfurt, Germany). Blanks containing assay buffer alone or with bovine serum albumin in the same concentration as the protein content in the cytosol-glycerol mixture were included.

To be sure that the supernatants are free of mitochondrial ornithine-2-oxo-acid aminotransferase²) which can also release CO_2 from ornithine via the glutamate-2-oxo-glutarate pathway the activity of the first 51 enzyme assays were performed also in the presence of final concentrations of 10 µmol/l aminooxyacetate which inhibits the aminotransferase activity without affecting the measurement of ornithine decarboxylase (30). Ornithine decarboxylase activity was also measured in final concentrations of 56 µmol/l α -difluoromethylornithine (Merrell Dow Pharma, Rüsselsheim, Germany), an irreversible inhibitor of ornithine decarboxylase (120 biopsies) (31). Furthermore, the ornithine decarboxylase activity was assayed in the presence of GTP in final concentrations of 70 µmol/l (114 biopsies). This is a sensitive method to detect a qualitatively different ornithine decarboxylase isoform (12, 13, 32). One unit of ornithine decarboxylase activity is defined as the amount releasing 1 nmol of CO_2 from *L*-ornithine per hour at 37 °C. Specific activity is expressed as nmol CO_2 per hour per mg protein, measured in the cytosol-glycerol fractions. Protein analyses were performed in

duplicate as described by *Bradford* (33) using bovine serum albumin as a standard.

Results

We examined 120 regional colonic mucosal biopsies, all obtained at standard conditions, for determination of ornithine decarboxylase activity. The enzyme analyses were performed within three weeks. During this time there was no loss of enzyme activity. The ornithine decarboxylase activity in the cytosol-glycerol mixtures remained stable. Some of the extracts (n = 29) we have tested several months later still showed the same results (tab. 1).

Enzyme determination (2 replicates) of the same cytosol-glycerol fractions was done with an intra-assay coefficient of 5.0% in all 120 biopsies, respectively. Different procedures of the supernatant as ultracentrifugation and dialysis did not alter the results. The differences of the ornithine decarboxylase activity of the 46 320 g supernatant and the 100 000 g supernatant were 4.0%. The ornithine decarboxylase activity of the non-dialyzed and the dialyzed cytosol supernatant also showed no significant differences (2.5%). Due to the lacking effects of ultracentrifugation and dialysis we limited this procedures to the first five specimens. The addition of bovine serum albumin to our assay blank in the same concentration as the protein content in the cytosol-glycerol mixture (about 30 µg protein) showed the same result as compared with the assay buffer alone (mean 49 counts/min without bovine serum albumin, 52 counts/min with bovine serum albumin, 5 cytosol-glycerol-fractions tested).

The addition of aminooxyacetate to the first 51 assays of specimens from all six segments from proximal to distal colon showed no difference to those assays without aminooxyacetate (-3.5% to + 2.5%). Thus, our cytosol-glycerol fractions were free from the non-specific

Tab. 1 Effect of storage (-20 °C) of cytosol-glycerol mixture from different biopsies on ornithine decarboxylase activity shown in 9 representative experiments

	Ornithine decarboxylase activity		
	fresh (1 day)	2 weeks	months
Sigma	0.67	0.66	
Caecum	0.29		0.27 (3 months)
Rectum	0.29	0.28	0.26 (6 months)
Transverse	0.51	0.53	0.51 (2 months)
Ascending	0.24		0.25 (1 month)
Rectum	0.32	0.30	
Rectum	0.58	0.61	
Rectum	0.61	0.57	
Rectum	0.28		0.29 (12 months)

Ornithine decarboxylase values are given as $^{14}\text{CO}_2$ liberated in nmol/h · mg protein

ornithine decarboxylase independent formation of $^{14}\text{CO}_2$ from DL -[1- ^{14}C]ornithine by the mitochondrial aminotransferase.

An incubation time longer than 30 min after acidifying the incubation mixture did not influence the results (10 cytosol-glycerol fractions tested).

The mean ornithine decarboxylase levels of all our subjects given as CO_2 liberated ranged from 0.26 ± 0.08 nmol/h · mg protein to 0.44 ± 0.16 nmol/h · mg protein and showed a slight increase according to the localization from proximal to distal colon with a considerable variation within each regional group. As also shown in table 2 there were no differences between sex and age.

Ornithine decarboxylase in normal mucosa was not stimulated by GTP. The ornithine decarboxylase activity of the specimens with GTP was on an average 1.01 fold of that without GTP (range from 0.97 to 1.06), measured in 114 biopsies. α -Difluoromethylornithine reduced the ornithine decarboxylase activity in all 120 biopsies by an average of 97.1% (96.5%–97.5%).

Discussion

We described a level of ornithine decarboxylase activity for normal human subjects whose age overlap with the risk for colorectal cancer. The determination of ornithine decarboxylase activity might be important for the detection of malignant and premalignant diseases of the colon. There are a lot of methodical discrepancies described in different papers (1, 25, 26, 28, 29). Different methods and procedures can significantly influence the results. As we could show in our standardized assay, ornithine decarboxylase activity can be measured with reasonable precision and reproducibility.

The addition of glycerol, pyridoxal-5-phosphate and EDTA to the cytosolic supernatant has a protective ef-

fect on the storage of the extract if frozen for months at -20°C (28, 29). As we could show in some of our experiments, it is not necessary to perform an ultracentrifugation of the homogenate. On the other hand the centrifugation procedure described by Garewal et al. (34): 5 min at 10 000 g, does not seem sufficient to eliminate all mitochondria. Therefore, mitochondrial ornithine decarboxylase activity and especially mitochondrial aminotransferase activity can influence the results. Due to our measurements in presence and absence of aminoxyacetate, an inhibitor of mitochondrial aminotransferase, we could show that our procedure is sufficient and that our results reflect the activity of the cytosolic active ornithine decarboxylase only.

Dialysis of the homogenate was suggested for the elimination of endogenous ornithine, or the small protein antizyme (35) which possibly could dilute the radioactive assay or inhibit the reaction. As we could show, there was no difference between the dialyzed and the non-dialyzed extracts. Thus, these products are negligible and do not play an important role in the ornithine decarboxylase assay.

The addition of bovine serum albumin to our assay in the same concentration as the protein content in the cytosol-glycerol mixture showed the same result as compared with the assay buffer alone. The protein concentration of about 30 μg in our standardized enzyme assay has been high enough for reproducible results. This is also different to the ornithine decarboxylase assay used by Garewal et al. (34).

An α -difluoromethylornithine corrected control of the ornithine decarboxylase assay is often recommended. The addition of α -difluoromethylornithine as a "background" sample may be acceptable in the ornithine decarboxylase assay of normally appearing mucosa. But using an ornithine decarboxylase assay with extracts from cancer mucosa which is often less inhibited

Tab. 2 Specific ornithine decarboxylase activity from proximal to distal normal colonic mucosa in 20 subjects and differentiated by sex and age

	Ornithine decarboxylase activity					
	Caecum	Ascending	Transverse	Descending	Sigmoid	Rectum
All (n = 20)	0.26 ± 0.08	0.27 ± 0.11	0.31 ± 0.14	0.35 ± 0.18	0.40 ± 0.20	0.44 ± 0.16
Men (n = 9)	0.23 ± 0.08	0.28 ± 0.11	0.35 ± 0.14	0.40 ± 0.21	0.47 ± 0.19	0.48 ± 0.15
Women (n = 11)	0.28 ± 0.08	0.26 ± 0.11	0.27 ± 0.14	0.30 ± 0.16	0.33 ± 0.20	0.41 ± 0.17
<50 years (n = 10)	0.25 ± 0.07	0.24 ± 0.12	0.26 ± 0.12	0.29 ± 0.15	0.36 ± 0.23	0.42 ± 0.15
≥ 50 years (n = 10)	0.26 ± 0.10	0.30 ± 0.08	0.38 ± 0.15	0.42 ± 0.21	0.44 ± 0.17	0.46 ± 0.18

Mean values of ornithine decarboxylase $\pm$ standard deviation are given as $^{14}\text{CO}_2$ liberated in nmol/h · mg protein

by α -difluoromethylornithine ((13), and our unpublished data), an α -difluoromethylornithine corrected control would give an uncorrect "background" reading. The α -difluoromethylornithine inhibition of 97.1% is also an indicator for the homogeneity of the ornithine decarboxylase measured in normal mucosa. The remaining activity is the result of unspecific decarboxylases. The lower α -difluoromethylornithine inhibition rate which was found in some carcinoma tissues (15) was not detectable in our specimens of normal mucosa.

Until now little is known about the intra- and inter-individual variability of ornithine decarboxylase in normal subjects. Our study shows a slight increase of ornithine decarboxylase levels from the caecum towards the rectum. This effect was detectable in 17 of our 20 patients, two showed no increase and one a slight decrease. The reason for this increase remains unclear, an elevated cell

turnover in the distal parts of the colon might play a role. No difference according to age and sex could be found. In contrast to our findings the results of Hixson et al. (23, 36) showed no variation in the ornithine decarboxylase activity with bowel location. He measured the ornithine decarboxylase activity in apparently unaffected colorectal mucosa but with known neoplastic lesions. The interpretation as "normal" colonic mucosa must therefore be questionable.

The presence of a GTP activatable ornithine decarboxylase which is described in carcinoma tissue (12, 13, 32) could not be found in normal colonic mucosa.

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